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Two Structural Variants of Nek2 Kinase, Termed Nek2A and Nek2B, Are Differentially Expressed in *Xenopus* Tissues and Development

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Nek2 kinase, a NIMA-related kinase, has been suggested to play both meiotic and mitotic roles in mammals, but its function(s) during development is poorly understood. We have isolated here cDNAs encoding a *Xenopus* homolog of mammalian Nek2 and have shown that *Xenopus* Nek2 has two structural variants, termed Nek2A and Nek2B. Nek2A, most likely a C-terminally spliced form, corresponds to the previously described human and mouse Nek2, while Nek2B is most probably a novel, C-terminally unspliced form of Nek2. As a consequence of this (probable) alternative splicing, Nek2B lacks the C-terminal 70-amino-acid sequence of Nek2A, which contains a PEST sequence (or a motif for rapid degradation). Western blot analysis reveals that Nek2A is expressed predominantly in the testis (presumably in spermatocytes) and very weakly in the stomach and, during development, only after the neurula stage. By contrast, Nek2B is expressed mainly in the ovary and in both primary and secondary oocytes and early embryos up to the neurula stage. These results suggest that Nek2A and Nek2B may play both meiotic and mitotic roles, but in a spatially and temporally complementary manner during *Xenopus* development, and that Nek2B, rather than Nek2A (or the conventional form of Nek2), may play an important role in early development. We discuss the possibility that a counterpart of *Xenopus* Nek2B might also exist and function in early mammalian development.

Key Words: **alternative splicing; cell cycle; development; meiosis; mitosis; Nek2; *Xenopus*.**

INTRODUCTION

In all eukaryotic cells, entry into M phase of the cell cycle is controlled by maturation/M-phase-promoting factor MPF, which is a complex of Cdc2 kinase and cyclin B (Masui and Markert, 1971; Nurse, 1990; Murray and Hunt, 1993). However, activation of Cdc2 kinase alone is not sufficient to trigger mitosis in certain eukaryotic cells (Osmani *et al.*, 1991a; Stueland *et al.*, 1993). Thus, in the filamentous fungus *Aspergillus nidulans*, NIMA, a Ser/Thr-protein kinase, has long been known to be required, together with Cdc2 kinase, for the initiation of mitosis (Oakley and Morris, 1983; Osmani *et al.*, 1988, 1991a; Lu and Means, 1994). Consistent with its mitotic function, NIMA is expressed at high levels only in G2 and M phases (Osmani *et al.*, 1991b) and, structurally, has a large C-terminal regulatory domain, which possesses several sequence motifs such as those for rapid degradation and phosphorylation by Cdc2 (O'Connell *et al.*, 1994; Fry and Nigg, 1995; Pu and Osmani, 1995). Recent work shows that NIMA plays a role in the nuclear localization of cyclin B, either by promoting its nuclear import or by preventing its nuclear export (Wu *et al.*, 1998).

Many genes encoding protein kinases structurally related to NIMA have been cloned from mammals (Letwin *et al.*, 1992; Schultz and Nigg, 1993; Schultz *et al.*, 1994; Lu and Hunter, 1995). Among these, Nek2 is most closely related to NIMA and has been best characterized. Thus, human Nek2 shares 47% amino acid identity over the catalytic domain with NIMA and also has a putative C-terminal regulatory domain (Schultz *et al.*, 1994). Moreover, the substrate specificity *in vitro* and some biochemical properties of Nek2 are reminiscent of NIMA (Fry *et al.*, 1995). However, significant differences also exist between Nek2 and NIMA. For instance, unlike NIMA, Nek2 contains no Cdc2 phosphorylation site motifs in the C-terminal region (Schultz *et al.*, 1994), and its activity peaks in S and G2 phases in human cells (Fry *et al.*, 1995). Recent work also shows that, unlike NIMA (O'Connell *et al.*, 1994; Lu and

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Hunter, 1995), overexpression of Nek2 does not induce chromosome condensation in HeLa cells but, strikingly, induces a splitting of centrosomes, suggesting a role for human Nek2 in centrosome separation (Fry *et al.*, 1998a). Thus, despite its similarity to NIMA, Nek2 may have a different mitotic function from NIMA.

In mice, Nek2 has recently been implicated in meiosis of both male and female germ cells. Thus, mouse Nek2 is expressed at high levels in the testis and at low levels in the ovary (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998). In the testis, Nek2 is expressed primarily in pachytene and diplotene spermatocytes (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997), in which the kinase seems to localize to the nucleus, associating with meiotic chromosomes (Rhee and Wolgemuth, 1997). In the ovary, Nek2 is highly expressed in meiotically active oocytes at the pachytene stage (Tanaka *et al.*, 1997) and also seems to associate with chromosomes (Rhee and Wolgemuth, 1997). In addition to germ cells, however, mouse Nek2 is also expressed in early embryos as well as in several adult tissues with high mitotic activity, such as intestine and thymus (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998). In the embryo, Nek2 is expressed mainly in the brain and a part of spinal cord, tissues that are still proliferating (and also differentiating) (Tanaka *et al.*, 1997). These studies suggest a role for Nek2 in both meiosis and mitosis during vertebrate development. However, little is known of whether Nek2 plays any specific role, particularly a role in cell cycle regulation, during vertebrate development.

As a first step toward understanding the roles of NIMA-related kinases in cell cycle regulation and development, we have undertaken here to clone their *Xenopus* homologs and, consequently, have isolated two structural variants of *Xenopus* Nek2, termed Nek2A and Nek2B. Nek2A, the genuine homolog of mammalian Nek2, is expressed highly in the testis and moderately in embryos only after the neurula stage, while Nek2B, most probably a novel C-terminally unspliced form of Nek2, is expressed in the ovary and in oocytes and early embryos up to the neurula stage. These results suggest that the two Nek2 variants (probably splice variants) may function for both meiosis and mitosis, but in a spatially and temporally complementary manner during *Xenopus* development, and that Nek2B, rather than Nek2A (or the conventional form of Nek2), may play an important role in early development. We discuss the role of Nek2A and Nek2B in *Xenopus* development, and raise the possibility that a counterpart of *Xenopus* Nek2B might also exist and function in early mammalian development.

MATERIALS AND METHODS

cDNA Cloning

By comparison of the amino acid sequence of NIMA with those of other known NIMA-related kinases [*Neurospora* NIM-1 (Pu *et al.*, 1995), budding yeast kin3 (Jones and Rosamond, 1990), mouse Nek1 (Letwin *et al.*, 1992), and human Nek2 (Schultz *et al.*, 1994)], we first designed degenerate primers for PCR. The 5′ primers were derived from peptide sequences L/MK/RHP/ENIC [5′-(A/T/C)CTNAA(A/G)CA(T/C)CCNAA(T/C)ATNG-3′] in the kinase domain IV and YI/LMME/DYC [5′-TA(T/C)(A/T/C)TNG-TNATGGANTA(T/C)TG-3′] in the kinase domain V; the 3′ primers were from peptide sequences VK/QLGDFG [5′-CC(A/G)AA(A/G)TCNCCNA(A/G)(T/C)T(T/G)NAC-3′] in the kinase domain VII and V/IGTPY/FYM/L [5′-A(A/T/G)(A/G)TA(A/G)TCNCCNA(A/G)(T/C)T(T/G)NAC-3′] in the kinase domain VIII. By using these primers, PCRs were performed against a *Xenopus* ovarian cDNA library (in λgt10; Rebagliati *et al.*, 1985). Amplified DNA fragments were subcloned into the pBSII KS+ vector (Stratagene, La Jolla, CA), sequenced, and found to encode Nek2-like sequences. A fragment (483 bp) 5′ terminal to one of the Nek2-like sequences was then obtained by PCR using best match primers, which were derived from the initially amplified DNA fragment (5′-CTAGCTAGCATGCCGTCACGGGTCGAG-3′) and from the λgt10 vector (5′-GCTGGGTAGTCCCCACCTTT-3′). This fragment was labeled with ^{32}P and used as a plaque hybridization probe to isolate longer Nek2-like cDNAs from the ovarian cDNA library. About 8.1×10^5 independent phages were screened and 15 positive clones were isolated. After subcloning, cDNAs were sequenced by using the Applied Biosystems Model 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences determined were analyzed by the use of the DNASIS sequence analysis program (Hitachi software, Japan) and were found to encode *Xenopus* homologs (Nek2A and Nek2B) of mammalian Nek2.

Construction of Recombinant Plasmids and in Vitro Transcription and Translation

To subclone Nek2A and Nek2B cDNAs into a transcription vector, PCR was first performed by using a 5′ UTR oligonucleotide primer, CGGGATCCATGCCGTCACGGGTCGAG (for both Nek2A and Nek2B), and either a 3′ UTR oligonucleotide primer, CGGGATCCAGCGTTACATAAGCAAGTAAGA (for Nek2A), or another 3′ primer, CGGGATCCTTAGAATTTGCTCCATTCAT-TCC (for Nek2B); these primers contained an artificial *Bam*HI site. The amplified fragments were cut with *Bam*HI and then inserted into the *Bam*HI-cut pT7G(UK−) vector, a pIBI derivative of the transcription vector pSP64T (Furuno *et al.*, 1994). pT7G(UK−) recombinant plasmids thus obtained were cut singly with *Not*I and then *in vitro* transcribed into 5′-capped mRNAs by using the MEGAscript T7 kit (Ambion, TX). For *in vitro* translation, 1 μg of *in vitro*-transcribed mRNA was translated in 50 μl of a reticulocyte lysate (Amersham, UK) for 90 min at 30°C.

Antibodies

To generate antibodies against *Xenopus* Nek2B, a full-length coding region of the Nek2B cDNA was subcloned into the bacterial expression vector pET15bs-1, which contains a histidine tag. Bacterially produced Nek2B–His fusion proteins were affinity-purified by using the Protein Purification System (Qiagen, Germany) and injected into rabbits for immunization. Anti-Nek2B antibodies were affinity-purified by using the Affi-Gel 10 immunoaffinity chromatography (Bio-Rad, Richmond, CA).

Western Blot Analysis

Routinely, five frozen oocytes or embryos were homogenized on ice in 50 μl of an extraction buffer (Gerhart *et al.*, 1984) and centrifuged for 3 min at 2°C; the supernatant was diluted with an equal volume of 2× Laemmli's sample buffer and boiled for 5 min.

Various tissues were also obtained from adult *Xenopus* males and females and processed as above. A portion, equivalent to one oocyte (or embryo) or 20 μg of tissue protein, was subjected to SDS–PAGE to make a Western blot. The blot was probed with affinity-purified anti-Nek2B antibodies and detected by using enhanced chemiluminescence reagents (Amersham, UK), essentially as described previously (Furuno *et al.*, 1994).

Preparation of Oocytes and Embryos

Preparation and culture of oocytes and embryos and induction of oocyte maturation by progesterone were all described previously (Sagata *et al.*, 1989; Furuno *et al.*, 1994). Embryos were staged according to Nieuwkoop and Faber (1956).

RESULTS

cDNA Cloning of Two Structural Variants of Xenopus Nek2, Nek2A and Nek2B

To clone cDNAs encoding *Xenopus* homologs of NIMA-related kinases, PCRs were first performed toward a *Xenopus* ovarian cDNA library, by using degenerate oligonucleotide primers derived from NIMA and NIMA-related kinases (see Materials and Methods). Several short DNA fragments were amplified, and an ~500-bp fragment 5′ terminal to one of the fragments was isolated by PCR. Using this 5′-end fragment as a probe, we performed plaque hybridization toward the ovarian cDNA library, which yielded 15 positive clones. One of the 15 clones, capable of encoding 442 amino acids (Fig. 1A), had a strong sequence similarity throughout the entire sequence to human and mouse Nek2, with overall 71 and 72% identity at the amino acid level, respectively (see Fig. 2); this clone was thus thought to encode a genuine *Xenopus* homolog of mammalian Nek2. The other 14 clones (of various lengths) were identical in nucleotide sequence to the above single (or rare) clone throughout the long-ranging 5′-end half (including the noncoding region), strongly indicating that both the abundant and the rare clones were derived from a single gene (Fig. 1B). Interestingly, however, the abundant 14 clones had a 3′-end half that was completely different from that of the rare clone and, importantly, retained a canonical splice donor sequence (AG ↓ GTATGA) exactly at the site (positions 1167–1174) with which the sudden 3′-end sequence divergence (from the rare clone) began (Fig. 1B). (Instead, at the corresponding site, the rare clone had a putative splice junction sequence or AG ↓ A; see Fig. 1A.) These results strongly suggest that the abundant 14 clones encoded a C-terminally unspliced form of Nek2 (consisting of only 389 amino acids due to the premature termination shortly after the splice donor sequence), while the rare clone encoded a C-terminally spliced form of Nek2. Accordingly, hereafter, we refer to the rare clone (which corresponds to the conventional human and mouse Nek2) as Nek2A and the abundant clones (which probably encode a novel, C-terminally unspliced form of Nek2) as Nek2B.

Structure of Nek2A and Nek2B

Nek2A and Nek2B are predicted to be Ser/Thr-protein kinases of 52 and 46 kDa, respectively, and are overall 32 and 31% identical to *Aspergillus* NIMA (Osmani *et al.*, 1988), respectively. Structurally, NIMA and human (and mouse) Nek2 can be divided into two domains, an N-terminal catalytic domain and a C-terminal regulatory domain (Lu and Means, 1994; Schultz *et al.*, 1994). The regulatory domain of NIMA has several sequence motifs that are required for rapid degradation, nuclear localization, protein–protein interaction, and phosphorylation by Cdc2 kinase (O'Connell *et al.*, 1994; Fry and Nigg, 1995; Pu and Osmani, 1995). In human and mouse Nek2, two PEST sequences (for rapid degradation; cf. Rechsteiner and Rogers, 1996) are present in the C-terminal regulatory domain (Fig. 2), and they seem to be functional in cells (Rhee and Wolgemuth, 1997). *Xenopus* Nek2A (corresponding to human and mouse Nek2) also possesses a single PEST-like sequence in its C-terminal region, but Nek2B, having a much shorter and distinct C-terminal sequence, does not (Fig. 2). However, none of the motifs for nuclear localization and phosphorylation by Cdc2 kinase can be seen in Nek2A and Nek2B as well as in human and mouse Nek2.

Characterization of Antibodies against Xenopus Nek2

For the detection of Nek2A and Nek2B in *Xenopus* tissues and cells, first of all, we prepared polyclonal antibodies, termed N1 to N4, that were raised in rabbits against a bacterially produced Nek2B–His fusion protein and then affinity-purified (see Materials and Methods). To test whether these antibodies could recognize both Nek2A and Nek2B, Western blot analysis was first performed toward Nek2A and Nek2B proteins that were synthesized *in vitro* in a reticulocyte lysate. A protein of 52 kDa (a size of Nek2A) and a protein of 46 kDa (a size of Nek2B) were clearly detected with N3 antibody in the Nek2A mRNA- and Nek2B mRNA-translated reticulocyte lysates, respectively; importantly, no such proteins were detected in a control lysate that did not contain added mRNA (Fig. 3). Thus, the N3 antibody could recognize both Nek2A and Nek2B specifically. (As can be seen in Fig. 3, the N3 anti-Nek2B antibody consistently detected Nek2B more strongly than Nek2A, presumably because it recognized preferentially the C-terminus of Nek2B, which is distinct from that of Nek2A.) The other three antibodies could also recognize, more or less, both the 52- and the 46-kDa proteins specifically (data not shown). Thus, these results demonstrate that the N1 to N4 antibodies can all recognize specifically both Nek2A and Nek2B.

Tissue-Specific Expression of Nek2A and Nek2B

Having determined the specificities of anti-Nek2 antibodies, we then examined the expression patterns of Nek2A and Nek2B in many adult *Xenopus* tissues, including brain, heart, lung, stomach, intestine, liver, kidney, muscle, tes-

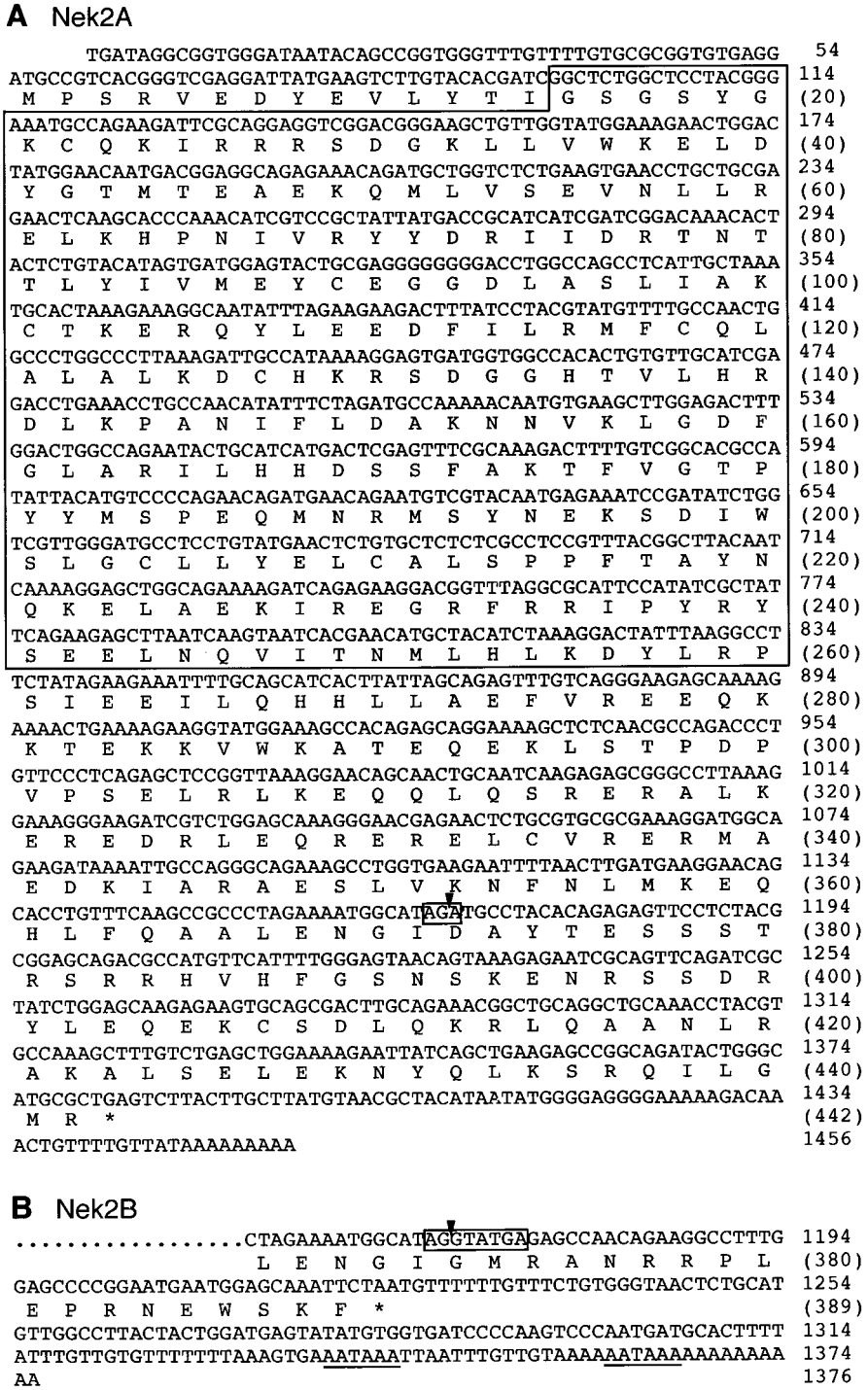

FIG. 1. Nucleotide and deduced amino acid sequences of Nek2A (A) and Nek2B (B). In (A), the kinase domain is boxed and the consensus sequence for a spliced site is indicated by the small box. In (B), only a relevant region (or a 3′-end region) is presented; the consensus sequence for a splice donor site is boxed and polyadenylation signals are underlined. The nucleotide sequence data are deposited with the DDBJ/EMBL/GenBank Data Libraries Under Accession Nos. AB019556 (for Nek2A) and AB019557 (for Nek2B).

tis, and ovary. In mice, high levels of Nek2 expression have been observed in the testis, with lower levels of expression in the ovary as well as in some other tissues (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998). Consistent with these observations, very high levels of a 52-kDa protein, which comigrated with *in vitro*-

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Xl. A MPSRVEDYEVLYTIGSGSYGKCQKIRRRSDGKLLVWKELDYGTMTEAEKQMLVSEVNLLR
Xl. B ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
human ::::A::::::::::T::::R::::::K::::::::::::::::::::::::::::::::
mouse ::::::::::::HS::T::::R::::::K::::I:::::::::S:::V::::::::::::::

Xl. A ELKHPNIVRYYDRIIDRTNTTLYIVMEYCEGGDLASLIAKCTKERQYLEEDFILRMFCQL
Xl. B ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
human ::::::::::::::::::::::::::::::::::::::V:T:G:::::::D:E:V::VMT::
mouse ::::::::::::::::::::::::::::::::::::::V:S:G::D::::::E:V::VMT::

Xl. A ALALKDCHKRSDGGHTVLHRDLKPANIFLDAKNNVKLGDFGLARILHHDSSFAKTFVGTP
Xl. B ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
human T::::E::R:::::::::::::::::V:::G:Q:::::::::::::N::T::::::::::
mouse T::::E::R:::::::::::::::::V:::S:H:::::::::::::N::T::::::::::

Xl. A YYMSPEQMNRMSYNEKSDIWSLGCLLYELCALSPPFTAYNQKELAEKIREGRFRRIPYRY
Xl. B ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
human ::::::::::::::::::::::::::::::::M:::::FS:::::G:::::K::::::::
mouse ::::::::SCL::::::::::::::::::::::M:::::F::::::G::::::::::::::

Xl. A SEELNQVITNMLHLKDYLRPSIEEILQHHLLAEFVREEQKKT-EKKVWKATEQEKLSTPD
Xl. B ::::::::::::::::::::::::::::::::::::::::::-::::::::::::::::::
human :D:::EI::R::N::::H:::V::::ENP:I:DL:AD::RRNL:RRGRQLG:P::SQDSS
mouse :DG::DL::R::N::::H:::V::::ESP:I:DM:A:::RRNL:RRGRRSG:PS::PDSS

Xl. A PVPSELRLKEQQLQSRERALKEREDRLEQRERELCVRERMAEDKIARAESLVKNFNLMKE
Xl. B ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
human ::L:::K:::I:::E::::::A::E::::K:Q:::::::L::::L::::N:L::YS:L::
mouse ::L:::K:::R:::D::Q::RA:::I:::K:::::I:::L::::L::::::M::YS:L::

Xl. A QHLFQAALENGIDAYTESSSTRSRRHVHFGSNSKEN--RSSDRYLE---QEKCSDLQKRL
Xl. B ::::::::::::GMRANRRPLEPRNEWSKF*
human RKFLSL:SN--PELLNLP::-VIKKK:::SGE::::IM::ENSESQLTSKS::K::K:::
mouse HR:LCL:GG--PE-LDLP::-AMKKK:::HGE::::TA::ENSESYLA-KS::R::K:::

Xl. A QAANLRAKALSELEKNYQLKSRQILGMR*

human H::Q:::Q:::DI:::::::::::::::*
mouse H::Q:::Q::ADI:::::::::::::::*
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FIG. 2. Comparison of the predicted amino acid sequences of *Xenopus* Nek2A (X1. A) and Nek2B (X1. B) with those of human (Schultz *et al.*, 1994) and mouse Nek2 (Rhee and Wolgemuth, 1997). Amino acid residues identical to those of *Xenopus* Nek2A are indicated by two dots (:). The potential spliced site in Nek2A is indicated by the arrowhead. PEST-like sequences are underlined.

synthesized Nek2A, were detected in the testis by using N3 antibody (Fig. 4). A similarly sized protein was also detected, albeit at much lower levels, in the stomach (but not in the other tissues, at least under the present conditions). On the other hand, a 46-kDa protein, comigrating with *in vitro*-synthesized Nek2B, was detected predominantly in the ovary and relatively weakly in the testis, but not in the other tissues (Fig. 4). The 52- and 46-kDa proteins were also detected specifically in the relevant tissues with other anti-Nek2B antibodies, but not with preimmune antibodies (data not shown), strongly suggesting that they were Nek2A and Nek2B, respectively. Thus, these results indicate that the two Nek2 variants, Nek2A and Nek2B, are differentially expressed in *Xenopus* tissues, the former mainly in the testis and the latter in the ovary. It should be noted here that the detection of Nek2B, but not Nek2A, in the ovary is consistent with the relative abundance of their cDNAs in the ovarian cDNA library (see above).

Expression of Nek2A and Nek2B during Oocyte Maturation

In the mouse ovary, Nek2 expression is confined mainly to primary oocytes (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997). Because Nek2B was detected predominantly in the ovary, we next examined its expression in oocytes and during oocyte maturation, by using N3 antibody. In immature oocytes (arrested at prophase I), a 46-kDa protein (most likely Nek2B) was readily detected (Fig. 5). After treatment of the oocytes with progesterone (to induce maturation), the levels of the protein remained essentially the same during the later course of meiosis I (including the stage of germinal vesicle breakdown), but then increased about twofold during meiosis II or in mature oocytes (Fig. 5). This 46-kDa protein was also detected with other anti-Nek2B antibodies, and its increase in abundance during meiosis II was abolished specifically by preinjection of the oocytes with Nek2B antisense (but not sense) oligonucleotides (data not

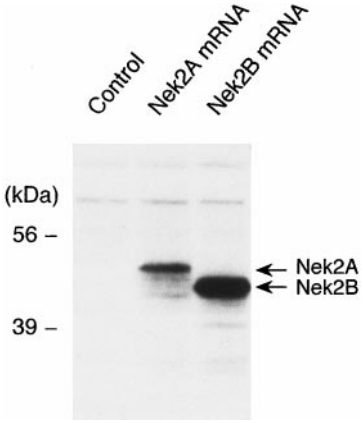

FIG. 3. Immunoblot analysis of *in vitro*-synthesized Nek2A and Nek2B proteins. Either 1 μg of Nek2A mRNA or 1 μg of Nek2B mRNA was translated in 50 μl of a reticulocyte lysate, and the total lysate (5 μl) was subjected to Western blot analysis with affinity-purified anti-Nek2B antibody. As a control, the same volume of a reticulocyte lysate with no added mRNA was used. Positions of Nek2A and Nek2B and sizes of protein markers (kDa) are indicated on the right and left sides, respectively.

shown), confirming the identity of the protein as Nek2B. Thus, clearly, Nek2B was present in immature oocytes and its levels increased significantly during *in vitro* maturation. In contrast with this, a protein of 52 kDa (or Nek2A) was not detected at all in immature oocytes or in maturing and mature oocytes (Fig. 5). This result is consistent with the lack of Nek2A expression in the ovary (see Fig. 4), but contrasts with the high expression of Nek2 (i.e., Nek2A) in mouse oocytes (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997).

Expression of Nek2A and Nek2B during Embryogenesis

Since Nek2B, but not Nek2A, was detected in mature oocytes, we further analyzed the expression patterns of Nek2A and Nek2B after fertilization or during embryogenesis. As in *in vitro*-matured oocytes, Nek2B was readily detected in *in vivo*-matured oocytes (or ovulated, unfertilized eggs) with N3 antibody. After fertilization, the levels of Nek2B increased slightly during the first few cell divisions and then remained constant up to the early blastula stage (embryonic stage 7) (Fig. 6). After this, however, the Nek2B levels decreased gradually during gastrulation and neurulation and then became undetectably low at the late neurula stage (stage 20) and later stages, at least up to the tadpole stage (stage 50). By contrast, Nek2A-like protein was not detected at all even after fertilization, at least until the late gastrula stage. Remarkably, however, concomitant with the disappearance of Nek2B, a protein of 52 kDa appeared at the early neurula stage (stage 14), and its levels increased and then remained constant after the late neurula stage, at least up to the tadpole stage (Fig. 6). This 52-kDa protein was also detected specifically with other anti-Nek2B antibodies but not with preimmune antibodies (data not shown), indicating that it was Nek2A. Thus, very interestingly, Nek2A was expressed only after the early neurula stage, and there was a switchover from Nek2B to Nek2A expression during the neurula stages. These results, together with the above results, suggest that, in *Xenopus* development, Nek2B may function in both oocytes and early embryos (up to the neurula stage), while Nek2A functions only in postneurula embryos. These results also suggest that the function of Nek2 gene during *Xenopus* embryogenesis may be regulated primarily at the level of alternative splicing. This idea would be interesting, since, in mouse embryos, only the conventional (probably C-terminally spliced) form of Nek2 or Nek2A has so far been detected (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998).

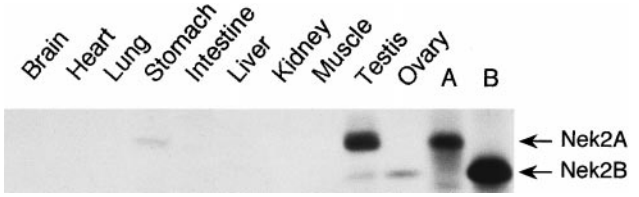

FIG. 4. Organ blot analysis of Nek2A and Nek2B. Total protein (20 μg) from each of various *Xenopus* tissues was analyzed by Western blot by using affinity-purified anti-Nek2B antibody. As controls for molecular weight standards, *in vitro*-translated Nek2A and Nek2B were used and are shown as A and B, respectively.

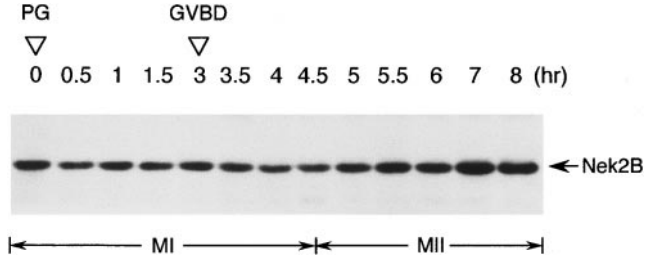

FIG. 5. Expression of Nek2A and Nek2B during oocyte maturation. Prophase-I-arrested immature oocytes were treated with progesterone (PG), and five oocytes each were sampled at the indicated times and analyzed by Western blot by using affinity-purified anti-Nek2B antibody. Each lane was loaded with proteins equivalent to one oocyte. Germinal vesicle breakdown (GVBD) occurred about 3 h after progesterone treatment. Periods of meiosis I (MI) and meiosis II (MII) are indicated (cf. Furuno *et al.*, 1994). Throughout maturation, Nek2A was not detected at all.

DISCUSSION

Among known mammalian protein kinases, Nek2 is structurally most closely related to *Aspergillus* NIMA (Fry

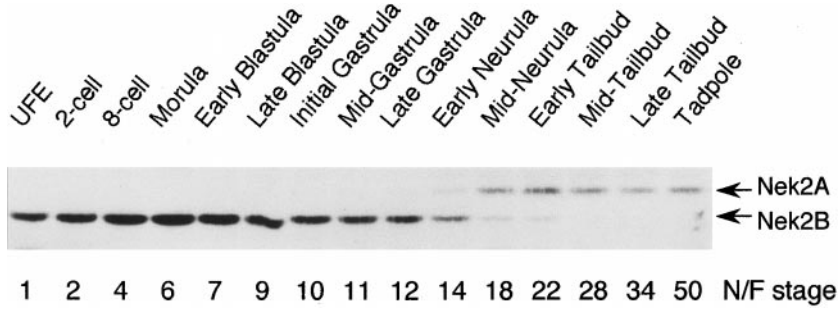

FIG. 6. Expression of Nek2A and Nek2B during embryogenesis. After fertilization, five embryos each were sampled at the indicated stages and analyzed as in Fig. 5. Each lane was loaded with proteins equivalent to one egg or embryo. UFE denotes an unfertilized egg. Nieuwkoop–Faber (N/F) stages are also indicated.

and Nigg, 1995), which is essential for entry into mitosis in the fungus (Morris, 1976; Osmani *et al.*, 1991a). Nek2 is expressed in various human cell lines (Schultz *et al.*, 1994) and is highly expressed in both primary spermatocytes and oocytes in mice (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998), suggesting a role in both mitosis and meiosis. Except for its recently reported role in centrosome separation in cultured human cells (Fry *et al.*, 1998a, b), the function(s) of Nek2 in any other cell types remains largely unknown.

We have isolated here cDNAs encoding a *Xenopus* homolog of mammalian Nek2 and have shown that *Xenopus* Nek2 has two structural variants, termed Nek2A and Nek2B. Nek2A, most likely a C-terminally spliced form, corresponds to the previously described human and mouse Nek2 (Schultz *et al.*, 1994; Rhee and Wolgemuth, 1997), while Nek2B is most probably a novel, C-terminally unspliced form of Nek2. As a consequence of this (probable) alternative splicing, Nek2B lacks the C-terminal 70-amino-acid sequence of Nek2A, which contains a PEST sequence or a motif for rapid degradation (cf. Rechsteiner and Rogers, 1996). So far, no splice variants of Nek2 have been isolated from any other species. Thus, our study is the first to suggest strongly that Nek2 has at least one splice variant other than the conventional form of Nek2 or Nek2A.

Interestingly, Nek2A and Nek2B were expressed differentially (if not totally) in adult *Xenopus* tissues: Nek2A protein was expressed very strongly in the testis (presumably in spermatocytes) and weakly in the stomach, but not in the ovary, while Nek2B was expressed predominantly in the ovary and weakly in the testis. (We cannot exclude the possibility that Nek2A or Nek2B was also expressed, but at undetectably low levels, in some other tissues.) Moreover and notably, the two Nek2 variants were also expressed differentially during development: Nek2A was expressed only in postneurula embryos, but Nek2B was expressed throughout oocyte maturation and early embryogenesis up to the neurula stage. Thus, Nek2A appeared to function mainly in spermatogenesis and late embryogenesis, while Nek2B functioned in oogenesis and early embryogenesis. Since Nek2 is thought to play a role in cell cycle control (cf. Fry *et al.*, 1995, 1998a; Rhee and Wolgemuth, 1997), our results would raise an intriguing model in which the two Nek2 variants may play both meiotic and mitotic roles, but in a spatially and temporally complementary manner, during *Xenopus* development (see Fig. 7). Moreover and importantly, our results suggest that Nek2B, rather than Nek2A (or the conventional form of Nek2), may play an important role in early development. To date, a number of genes involved in cell cycle control have been isolated and analyzed, but, in almost all cases, their functions (during development) are known to be regulated at the transcriptional and posttranslational levels (Norbury and Nurse, 1992; Morgan, 1995; Edgar, 1995). Our results suggest that *Xenopus* Nek2 gene function during development may be regulated basically at the posttranscriptional level or by alternative splicing.

In mice, Nek2 is highly expressed in the testis and embryos and, to a lesser extent, in the ovary as well as in some other tissues; in the testis Nek2 expression is restricted to spermatocytes, while in the ovary mainly to oocytes (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1997). Thus, as a whole, the expression

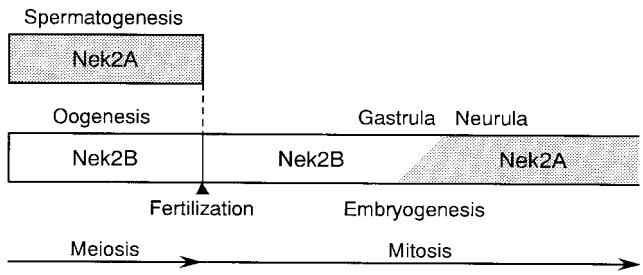

FIG. 7. Model for the function of Nek2A and Nek2B in *Xenopus* development. The model is a speculative one and is presented in the simplest form, based solely on the tissue-specific and developmental expressions of Nek2A and Nek2B. (Though not shown, Nek2B might also function, at least in part, for spermatogenesis, since it was also detected, but at low levels, in the testis; see Fig. 4). See text for details.

patterns of mouse Nek2 and *Xenopus* Nek2 (including both Nek2A and Nek2B) are very similar to each other. (*Xenopus* Nek2A in postneurula embryos might be expressed, at least in part, in neural tissues, since, in mouse embryos, Nek2 has been shown to be expressed mainly in the brain; Tanaka *et al.*, 1997.) Considering the mouse Nek2 expression in the ovary, oocytes, and early embryos, however, the apparent lack of *Xenopus* Nek2A (the genuine homolog of mouse Nek2) expression in the corresponding tissues and cells is somewhat curious. Formally, this may well be due to the expression of *Xenopus* Nek2B in those tissues and cells and hence due to the species-specific expression of Nek2 variants in them (in this case, Nek2B could compensate for the lack of Nek2A). However, it could also be due, at least in part, to the identity of the mouse Nek2 detected: at least part of the mouse Nek2 that was detected in the above tissues and cells might be, in fact, a mouse counterpart of *Xenopus* Nek2B, since the probes used to detect mouse Nek2 in the previous (histochemical) studies could also have detected mouse Nek2B transcripts or protein if it existed. [Moreover, the failure to detect Nek2B in the mouse ovary by RNA protection and Northern blotting (Tanaka *et al.*, 1997; Rhee and Wolgemuth, 1997) might be due to the fact that, unlike the *Xenopus* ovary, the mouse ovary consists mostly of somatic cells, which could contain only Nek2A (cf. Tanaka *et al.*, 1997).] Indeed, it seems possible for mammalians to have their own Nek2Bs, since both human and mouse Nek2 cDNAs have a putative splice junction sequence (AG ↓ A) exactly at the corresponding site of *Xenopus* Nek2A (cf. Schultz *et al.*, 1994; Rhee and Wolgemuth, 1997) and hence seem to be truly spliced at that site. Thus, although Nek2B might be specific to *Xenopus*, it could also be possible that a counterpart of Nek2B exists and functions in early mammalian development.

The differential occurrence of two Nek2 variants—most probably splice variants—in *Xenopus* tissues and development is intriguing. In principle, this could be caused by the different Nek2 splicing activities in different tissues and developmental stages. Whatever the precise mechanisms of the differential Nek2 splicing, however, the two Nek2 variants, once produced (though in different cells), may well undergo different regulations, such as those in stability and cellular localization, because they differ greatly structurally in their C-terminal regulatory regions. For instance, Nek2A has a PEST-like sequence in its C-terminal region, but Nek2B does not; this difference might result in a rapid, cell cycle-dependent degradation of Nek2A on one hand (as suggested for mouse Nek2; Rhee and Wolgemuth, 1997) and a stabilization of Nek2B on the other hand (which seems truly the case in oocytes and early embryos; our unpublished data; see also Figs. 5 and 6). Due to such different regulations, Nek2A and Nek2B might have some subtle (but significant) difference in their functions. To test this possibility, it will be important to determine whether the functions of Nek2A and Nek2B can be substituted for with each other during development.

Based on its chromosomal localization in spermatocytes and oocytes, mouse Nek2 has been assumed to play a role in chromosome condensation (Rhee and Wolgemuth, 1997). However, our preliminary results show that virtually all Nek2B proteins localize to the cytoplasm in *Xenopus* oocytes and that overexpression of Nek2A or Nek2B in oocytes, like overexpression of human Nek2 in cultured cells (Fry *et al.*, 1998a), cannot induce chromosome condensation (our unpublished data). On the other hand, based on its centrosomal localization and the ability of its overexpression to induce a splitting of centrosomes in cultured cells, human Nek2 has recently been suggested to function for centrosome separation (Fry *et al.*, 1998a,b). If this function is general for Nek2 in vertebrate cells, then, in *Xenopus*, Nek2A would function for centrosome separation principally in postneurula cells and spermatocytes, while Nek2B would do so in preneurula cells and oocytes. If so, the stability of Nek2B in early *Xenopus* embryos (see above) could account, at least in part, for the "autonomous" duplication and separation of centrosomes that are known to occur specifically in these early embryos (Gard *et al.*, 1990). Strikingly, however, it is well known that *Xenopus* oocytes, like oocytes of many other species including human, do not contain functional centrosomes (Gard, 1994; Navara *et al.*, 1995), suggesting some another noncentrosomal function(s) of Nek2B (and Nek2) in oocytes. [Indeed, even in cultured human cells, Nek2 is likely to have yet another function (in addition to the centrosomal function), since, in these cells, the majority of Nek2 proteins show a noncentrosomal localization (Fry *et al.*, 1998a).] Thus, the function(s) of Nek2, whether general or specific in meiosis and mitosis in vertebrate cells, is still largely enigmatic. Our findings on the existence and (differential) developmental expression of two Nek2 variants in the experimentally feasible *Xenopus* system (Fig. 7) would facilitate elucidation of the Nek2 function(s) in cell cycle regulation and development in vertebrates.

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